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14. ABSTRACT *Purpose*: We are testing the hypothesis that PRL-array-containing cell lines can be used effectively to identify novel molecular players in ERα transcriptional activation and repression, and in determining temporal patterns of function of all known ERα-coregulators.
Scope: We are using special cell lines containing chromosomally integrated arrays of estrogen-responsive reporters combined with automated and other types of single cell microscopic, imaged based assays. This systems-biology level approach will integrate functional data from multiple readouts at a single cell level for ERα and CoRs including 1) nuclear targeting, 2) promoter array occupancy, 3) large scale chromatin modeling, 4) histone modifications, and 5) mRNA synthesis.
Major Findings: ERα is differentially recruited to the PRL-array in HeLa cells by estrogen or epidermal growth factor, two critical signaling modalities in breast cancer. Transcription and chromosomal condensation mediated by ERα can be uncoupled. ERα mediated accumulation of reporter transcripts has differential periodicity in response to E2 or EGF. E2-induces a novel 24-hour cyclical pattern of accumulation of mRNA. Significant progress in technology and analytical techniques and modification of planned reporter constructions will facilitate achieving our proposed objectives.

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Introduction.

Estrogen receptor α (ERα) has a variety of central physiological roles including mammogenesis where it plays a pivotal role in mediating ovarian-directed developmental cues. Along with these ontological functions, it also plays crucial roles in the development of breast cancer, and in some way, all endocrine-based treatments target ER_ (1). ER_ mediates estrogen responsiveness via classical genomic (nuclear transcription factor) and recently reported nongenomic (plasma membrane signaling) effects. In our project, we are focusing on its genomic actions in response to agonists (estradiol, E2, or epidermal growth factor, EGF) and antagonists (4-hydroxy tamoxifen, 4HT, or ICI 182,780, ICI). In this capacity, ER_ regulates transcription of target genes in conjunction with coactivators and corepressors (coregulators, CoRs (2)). Pathologies are associated with ~160 of the 270 CoRs listed on www.nursa.org/, strongly suggesting that their role in breast cancer will be pervasive. An important task is to figure out which of these are important specifically for ER_.

Since coactivators and corepressors have opposing actions, their balance determines the ultimate biological effect of ligand-responsiveness mediated by the ER_. Coactivators function at multiple levels from transcription to translation (3). Based on current models, ER_-coactivator multisubunit complexes are likely to be engaged in co-regulating the initiation, elongation, and/or termination of transcription, in addition to processing nascent array transcripts for export out of the nucleus as mature mRNAs. Since ER_-mediated transcription response is our interest, we focusing on their activities at a single cell level, using a recently developed integrated chromosomal locus containing transcription units regulated through estrogen responsive elements (ERE), and modeled after those in the enhancer of the prolactin gene (4).

Our PRL-HeLa cell line bears a chromosomally integrated array of ~200 copies of an ERE-rich binding site element (e.g., super-enhancer) linked the PRL enhancer/promoter. This upstream segment is transcriptionally fused to the dsRED2-SKL reporter (4). The PRL-array is a powerful model system to test multiple aspects of ER_ function in the context of direct (ligand binding) and indirect (growth factor) signaling. In our initial report on the PRL-HeLa line (4), we showed that the integrated array of reporters is visible as two fluorescence foci when GFP-ER_ is exogenously expressed, expands upon agonist (E2) treatment, and contracts dramatically upon treatment with antagonists (4HT and ICI); when fully decondensed, the foci often merge into a larger structure. These dynamics change in concert with corresponding increases and decreases in array-associated reporter mRNA, respectively. Thus, while the PRL locus is engineered in HeLa cells, there is a very strong preservation of endocrinological regulation that is seen in the animal. In agreement with the "hit and run" model of nuclear receptor action in live cells (5), photobleaching studies provided direct evidence of very transient ER_-array interactions (~4-6s), and revealed ligand-dependent changes in receptor k_{off} (4). Importantly for this application, we also documented the association of RNA polymerase II and several coactivators known to be important for ER_ genomic action.

We are investigating the spatiotemporal pattern of ER_ CoR association with the existing PRL-HeLa, and developing multicopy transcription unit arrays in an ERα-negative breast cancer (MCF7-C4/C412) and non-tumorigenic mammary epithelial (MCF10A) lines. Data obtained will be crucial to an improved systems biology level understanding (afforded by multiplexed single cell analyses) of the temporal responses of ER_ to two physiological and breast cancer related agonists and antagonists.

Our Hypothesis remains: High throughput microscopy (HTM) in conjunction with multicopy transcription unit array-containing cell lines can effectively identify novel molecular players in ERα transcriptional activation and repression, and will determine functionally relevant spatiotemporal patterns of known coregulators. This systems biology level approach will integrate functional data from multiple readouts ***at a single cell level*** for ER_ and CoRs including 1) nuclear targeting, 2) promoter array occupancy, 3) large scale chromatin modeling, 4) histone modifications, and 5) mRNA synthesis.

Body – Work Accomplished.

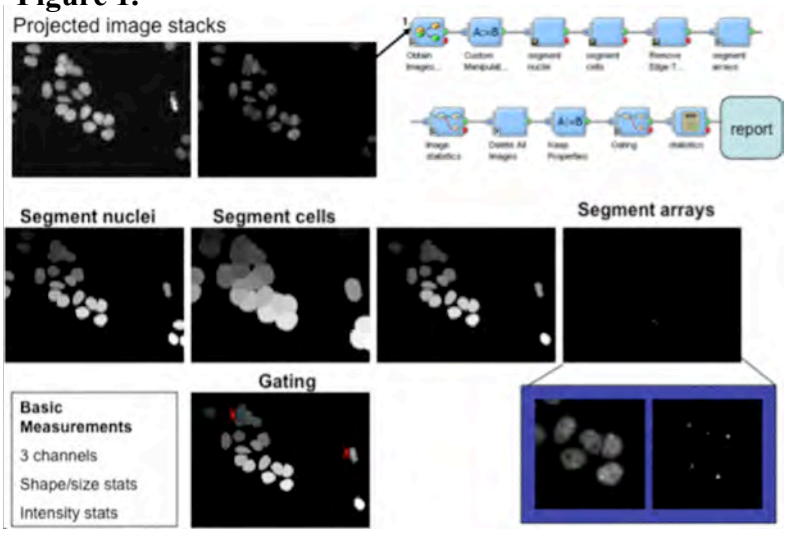
Task 1. To document the spatiotemporal pattern or ER_ CoR co-association with and dissociation from the ER_ targeted PRL array: (Months 1-18)

a. Immunofluorescence assays of CoR co-localization in response to ligand-dependent and –independent activation in PRL-HeLa. (Months 1-12)

b. FISH quantification of reporter mRNA, in response to ligand-dependent and –independent activation PRL-array reporter gene in PRL-PRL-HeLa. (Months 1-12)

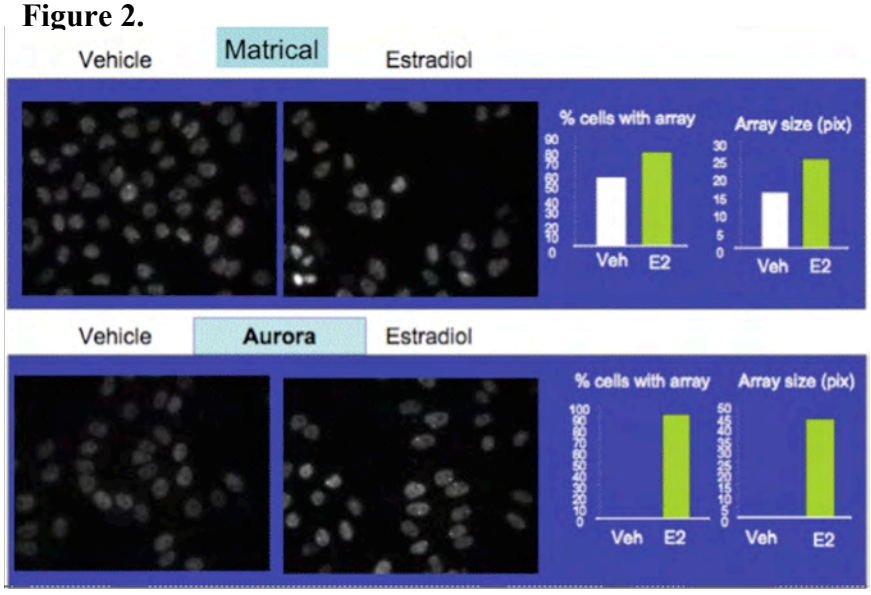
c. Measurements of large-scale chromosome (chromatin dynamics: decondensation or condensation) by in response to ligand-dependent and –independent activation in PRL-HeLa. (Months 1-12).

Figure 1.



Progress on this aim has been hampered by a need for a more powerful analytical tool to obtain statistically significant results. To effectively achieve the objectives of this task, it was necessary to focus on the development of new analytical methodology. Toward this end, we have adopted the Advanced Imaging Collection from Accelrys as a server client Image Analysis platform for enhanced measurement capabilities. In **Figure 1**, an analysis of image data using Pipeline Pilot is shown. The projected image stacks are taken and run through a series of image analysis protocols to segment cells into nuclear, cytoplamsmic and array compartments. A large number shape, size, intensity and texture measurements can be generated from these compartments, for up to 4 colors. This capability will greatly enhance our capability to achieve the objectives in the above tasks.

Figure 2.

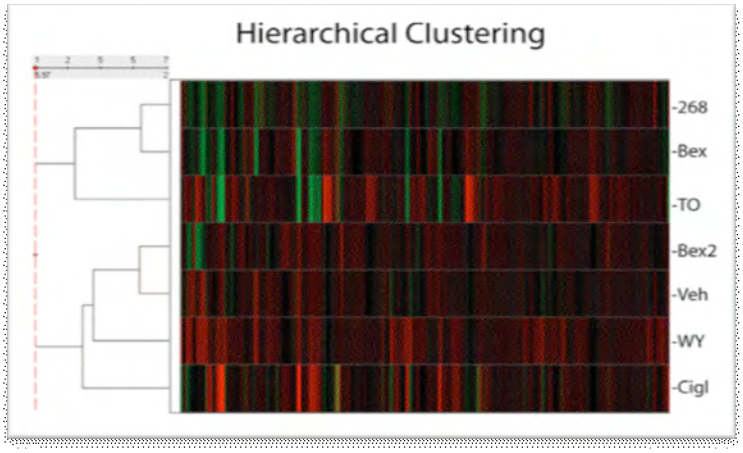


Progress was also hampered by a our discovery of xenobiotic estrogenic activity in our tissue culture plasticware. Our PRL-HeLa cells turned out to be excellent for assaying this activity, which we examined in plasticware from a variety of suppliers. Shown in Figure 2 are data using plates from two suppliers, Matrical and Aurora, which were assayed for estrogenic activity using double stable (Prl-HeLa +GFP-ER) "Sheila" cells. The percentage of visible arrays (ie., those associated with GRF-ER) is graphed on the right. In vehicle-treated cells grown in Matrical, but not in those from Aurora, there is a high percentage of cells demonstrating visible arrays. Our ER studies, and those of investigators will require a full understanding of the 'estrogenic' influence of the tissue culture plastic.

The development of "Sheila" cells was a significant accomplishment during this reporting period. Previously, to observed ER action at the PRL-array in HeLa cells, we had to transiently express GFP-ER

fusions. Shelia cells have been engineered to stably express physiological levels of GFP-ER in our PRL-HeLa line, thus eliminating the need for transfections, which will greatly expand the experimental possibilities for our Tasks shown above.

Figure 3.



Another advancement in data analysis is illustrated in **Figure 3**. All of the tasks in our project requires sophisticated image analysis routines that move well beyond descriptive biology into the realm of quantitative systems biology. This visual shows advances in our ability to quantify single cell responses to different treatment conditions using high throughput microscopy (HTM) and increasingly advanced usage of state-of-the-art image analysis tools. This figure shows that single cell data can be parsed in a dendogram that illustrates similarities in cellular responsiveness between six treatments of mammary epithelial cells (MCF-7) compared to vehicle. Here, 565 individual measurements were made for each of >150 cells per well (in triplicate) captured by HTM. For this study, based upon RXRα (retinoid X receptor), which has a significant role in fat metabolism) MCF-7 were treated with vehicle or the compounds listed. HTM-based measurements were made for RXRα expression level and localization (anti-RXRa, green), neutral lipid accumulation (LipidTOX, Texas red) and DNA content (DAPI, blue). Cigl = Ciglitazone (a PPARγ agonist), WY = WY14643 (a PPARα agonist), TO = T01317 (a LXR, liver X receptor, agonist), BEX = bexarotene (a RXR agonist, aka LGD1069 or Targretin), and LG268 = LG100268 (a more specific RXR agonist). These are all synthetic ligands to nuclear receptors that partner with RXR, some of them are potent and some are putative cancer preventive agents. The goal of this 'fingerprinting' experiment was to quantify cellular responsiveness using a systems biology level approach (e.g., functional/phenotypic parameters derived from DNA staining, nuclear/chromatin structure, cell/nuclear shape, subcellular/subnuclear distribution and trafficking of RXR, and cellular lipid content of MCF-7 cells, which could be used as biomarkers for a cancer preventive effect. Measurements were made by Pipeline Pilot (advanced imaging collection) and for Cluster Analysis and Principle Component Analysis (not shown). This type of advanced analysis will be critical for accomplishment of all of our aims.

Task 2. Generate new chromosomally integrated PRL- and pS2-based arrays in MCF7-C4/C4-12 breast cancer and MCF10A cells, and perform the assays in Task 1. (Months 1-24)

a. Construct cell lines with stable integration of PRL-based reporter plasmids and perform assays in Task 1: (Months 1-24)

   i. Transfect with existing PRL reporter plasmids and screen for stable lines expressing dsRED2-skl reporter. (Months 1-6).

   ii. Screen for visible arrays (Months 6-12)

   iii. Perform Task 1 assays, and determine copy number. (Months 6-24)

b. Construct cell lines with stable integration of pS2-based arrays and perform the assays as in Task 1. (Months 1-24)

   i. Construct pS2 based plasmids vectors. (Months 1-6)

   ii. Transfection, screening stable colonies with dsRED2-skl reporter expression, and screen positives for visible arrays. (Months 6-18)

   iii. Perform the Task 1 assays, and determine copy number. (Months 12-24)

c. Construct cell lines with stable integrations of the pS2-dsRED2-skl reporters DHFR amplification. (Months 1-24)

   i. Transfect, screen stable cell colonies for reporter expression, and screen positives for visible arrays. (Months 1-12)

   ii. Perform Task 1 assays, and determine copy number. (Months 12-24)

In this task, we have focused on the development of the PS2 promoter/reporter construct, which will be used to establish new breast cancer lines with a chromosomally integrated version of this reporter. A significant problem arose when we found that the promoter obtained from the Giguere laboratory were constitutive in our transcription assays in MCF7 cells expressing the estrogen receptor. We recloned it from human genomic DNA using high fidelity PCR, sequenced it to verify that all of the important functional elements (ERE, EGF sites and TATA core promoter sequences) were intact, added an 500bp of 5' flanking sequence, and assayed it using a mCherry reporter (more about this below). This construct was also constitutive, and was not inducible with E2 or repressed by tamoxifen in transient transfection assays.

To circumvent this significant problem, we have placed a far upstream enhancer element recently identified by Pan et al., (6) into our reporter constructs. Using PCR and standard cloning, the construct in **Figure 4** is currently being assembled for testing in both transient and stably tranfected MCF7 cells. Another recent report showed the importance of chromatin in hormone regulated PS2 reporter expression. Oduro, et al., (7) showed that PS2-Luciferase promoter was highly active constitutively in a cell line in which it was stably integrated into a chromosomal location, which bore a chromatin signature for active transcription. Our working model is that the PS2 ERE-containing enhancer in combination with the promoter ERE is required for full estrogen responsiveness, and that the PS2 enhancer will mitigate position (chromatin) effects when integrated into a chromosomal location. It is interesting that this enhancer in the PS2 gene is similar in position to that found in the prolactin gene, on which the PRL-HeLa line was designed (4).

Figure 4

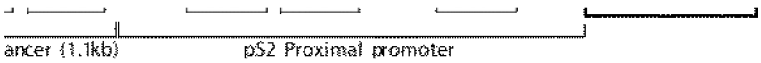
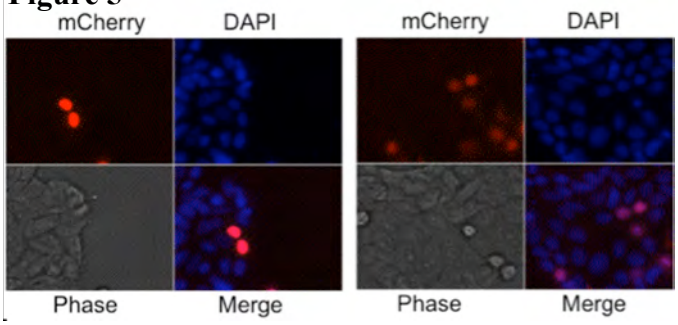


Figure 5



We have also obtained an improved version of mCherry for nuclear localization, courtesy of Fred Schaufele. This version has two copies of the mCherry reading frame, separated by a region containing a nuclear localization sequence. As you can see in **Figure 5**, this reporter is a predominantly partitioned to the nucleus, which will result in better quantification in our automated microscopic and other cell based assays.

In summary for this task, we expect to have MCF7 cell lines within 6 months, which be ready for the cell based experiments outline in the other Tasks above. The PRL arrays in MCF7 is a lower priority for us at present since this would be less biological relevant to breast cancer.

Task 3. Screen for novel ER_ coregulators using RNAi libraries and PRL-HeLa and PRL- and pS2-MCF7-C4/C4-12 and MCF10A array containing cells. (Months 1-24)

We are just beginning to obtain data in this set of experiments.

Key Research Accomplishments.

1. Made significant progress in implementing improved analytical capabilities needed for all of the assays involving automated microscopic experiments.
2. Discovered that plasticware has xenobiotic estrogenic activity, and that our Shelia cell line is a sensitive live cell technique for detecting this activity, which could benefit commercial laboratories as a method for xenobiotic estrogen testing in plastic products, and to the general academic research community especially those engaged in endocrine research.
3. Discovered that, contrary to published results, the version of the PS2 promoter used to test for estrogen regulation in MCF7 ER-containing cells is, in fact constitutively expressed.

Reportable Outcomes.

Our paper was published by *PLoS ONE.* It is available online at http://www.plosone.org/doi/pone.0002286

Title: Activation of Estrogen Receptor-_ by E2 or EGF Induces Temporally Distinct Patterns of Large-Scale Chromatin Modification and mRNA Transcription

Authors: Berno V, Amazit L, Hinojos C, Zhong J, Mancini MG, Sharp ZD, and Mancini MA.

Abstract: Estrogen receptor-_ (ER) transcription function is regulated in a ligand-dependent (e.g., estradiol, E2) or ligand-independent (e.g., growth factors) manner. Our laboratory seeks to understand these two modes of action. Using a cell line that contains a visible prolactin enhancer/promoter array (PRL-HeLa) regulated by ER, we analyzed ER response to E2 and EGF by quantifying image-based results. Data show differential recruitment of GFPER to the array, with the AF1 domain playing a vital role in EGF-mediated responsiveness. Temporal analyses of large-scale chromatin dynamics and accumulation of array-localized reporter mRNA over 24 hours showed that the EGF response consists of a single pulse of reporter mRNA accumulation concomitant with transient increase in array decondensation. Estradiol induced a novel cyclical pattern of mRNA accumulation with a sustained increase in array decondensation. Collectively, our work shows that there is a stimuli-specific pattern of large-scale chromatin modification and transcript levels by ER.

The following paper is submitted *Molecular Endocrinology,* and shows that the estrogen receptor research community has accepted our PRL-HeLa cell system as a valuable too to address novel and important question regarding this transcription factor and its signaling system and their role in breast cancer.

Title: Anti-estrogen resistance mediated by cyclin D1 overexpression

Authors: Wilbert Zwart; Mariska Rondaij; Kees Jalink; Z. Dave Sharp; Michael A Mancini; Jacques Neefjes; Rob Michalides

Abstract: Early diagnosis of resistance to endocrine treatment of breast cancer is essential, since alternative anti-estrogens are available for further treatment. We have shown before that anti-estrogens can be classified for the effects of defined phosphorylation sites in ER_ that, as the result of PKA and/or MAPK activity, are responsible for resistance to that particular anti-estrogen. In this study, we demonstrate that overexpression of cyclin D1, being a specific cofactor of ER_, renders ER_ resistant to a new 3rd generation anti-estrogen, arzoxifene. Arzoxifene is an effective growth inhibitor of ER_ positive human breast cancer cells, including tamoxifen resistant tumors and has therefore additional treatment value to the commonly used drug tamoxifen.

Here, we show that overexpression of cyclin D1 alters the conformation of ER_ in the presence of arzoxifene (by FRET and FLIM). Then ER_ still recruits RNA polymerase II to an EREcontaining promoter, and is responsible for transcription of an ER-dependent reporter gene. Cyclin D1overexpression then drives arzoxifene-stimulated proliferation of MCF-7 cells. Overexpression of cyclin D1 thus converts arzoxifene from an ER_-antagonist into an ER_-agonist. These findings of anti-estrogen specific modifications of ER_ being responsible for resistance to particular anti-estrogens may be of the utmost clinical relevance. When applicable for clinical testing, they indicate resistance to a particular anti-estrogen in the treatment of breast cancer and provide guidelines for the development of new anti-estrogens. The results further indicate that subtle changes in the conformation of ER_ upon binding of anti-estrogens are at the basis of resistance to anti-estrogens.

Although the following paper was not directly supported by our BCRP Synergy grant, it is important because it is a significant step toward application of the automated cell based imaging and novel analytical tools in a systems level analysis of hormone action. Using patient-derived mutations in the AR, we provided proof-of-principle evidence for an eventual application in personalized medicine. It is thus relevant to our overall research program, including our DOD sponsored projects, as we strive to translate our techniques and results toward more effective diagnostics and/or therapeutics for breast cancer. This paper is in press at *PLoS One*.

Title: Androgen receptor functional analyses by high throughput imaging: determination of ligand, cell cycle, and mutation-specific effects

Authors: Adam T. Szafran, Maria Szwarc, Marco Marcelli* and Michael A. Mancini

Abstract. *Background*: Understanding how androgen receptor (AR) function is modulated by exposure to steroids, growth factors or small molecules can have important mechanistic implications for AR-related disease therapies (e.g., prostate cancer, androgen insensitivity syndrome, AIS), and in the analysis of environmental endocrine disruptors.

Methodology/Principal Findings. We report the development of a high throughput (HT) image-based assay that quantifies AR subcellular and subnuclear distribution, and transcriptional reporter gene activity on a cell-by-cell basis. Furthermore, simultaneous analysis of DNA content allowed determination of cell cycle position and permitted the analysis of cell cycle dependent changes in AR function in unsynchronized cell populations. Assay quality for EC50 coefficients of variation were 5-24%, with Z' values reaching 0.91. This was achieved by the selective analysis of cells expressing physiological levels of AR, important because minor over-expression resulted in elevated nuclear speckling and decreased transcriptional reporter gene activity. A small screen of AR-binding ligands, including known agonists, antagonists, and endocrine disruptors, demonstrated that nuclear translocation and nuclear "speckling" were linked with transcriptional output, and specific ligands were noted to differentially affect measurements for wild type versus mutant AR, suggesting differing mechanisms of action. HT imaging of patient-derived AIS mutations demonstrated a proof-of-principle personalized medicine approach to rapidly identify ligands capable of restoring multiple AR functions. Conclusions/Significance. HT imaging-based multiplex screening will provide a rapid, systems-level analysis of compounds/RNAi that may differentially affect wild type AR or clinically relevant AR mutations.

Summary: In our published work, we have shown that the ER_ is differentially recruited to the PRL-array in HeLa cells depending upon whether they were treated with estrogen or epidermal growth factor, two critical signaling modalities in breast cancer. We also showed that transcription and chromosomal condensation can be uncoupled. Importantly, we also show that over a twenty-four hour time span, the ER_ mediated accumulation of reporter transcripts at the chromosomal array loci has differential periodicity depending upon if it is E2 or EGF induced. E2 induced a novel cyclical pattern of accumulation in associated with a decondensed array. Our

other progress in technology and analytical techniques outlined above should speed up findings in our current objectives. We have had to modify our original plans for the estrogen-regulated PS2 promoter constructions due to unforeseen problems with constitutive expression, which we are optimistic will be effective. Overall, we feel that our two labs are working synergistically very well to achieve our goal to better understand the role of the estrogen receptor in development and progression of breast cancer.

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